PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/88, A61K 7/135, 7/48, D06L 3/00, G01N 33/53, 33/577 — A1

(11) International Publication Number: WO 97/35998

(43) International Publication Date: 2 October 1997 (02.10.97)

(21) International Application Number: PCT/US97/04752

(22) International Filing Date: 27 March 1997 (27.03.97)

(30) Priority Data:
08/623,364 28 March 1996 (28.03.96) US

(60) Parent Application or Grant
(63) Related by Continuation
US Not furnished (CIP)
Filed on Not furnished

(71) Applicant *(for all designated States except US)*: TRUSTEES OF BOSTON UNIVERSITY [US/US]; 147 Bay State Road, Boston, MA 02215 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: GILCHREST, Barbara, A. [US/US]; 67 Walnut Place, Brookline, MA 02146 (US). PARK, Hee-Young [US/US]; 107 Marlboro Street, Chelsea, MA 02150 (US).

(74) Agents: GRANAHAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02173 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHODS OF MODULATING MELANIN SYNTHESIS

(57) Abstract

Methods are described whereby vertebrate skin, hair, wool or fur may be lightened, or darkened, in color by administration of a substance, e.g., peptide, antibody, antibody fragment or DNA sequence encoding a peptide, that modulates the protein kinase C-beta-mediated activation of tyrosinase, the rate-limiting enzyme in melanogenesis.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

METHODS OF MODULATING MELANIN SYNTHESIS

RELATED APPLICATIONS

This application is a continuation-in-part of prior Serial No. 08/623,364 filed March 28, 1996, the teachings of which are hereby incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

Cosmetically displeasing hyperpigmentation of the skin, due to increased melanin content in melanocytes, and the surrounding keratinocytes can result from burns, or other injuries, and is also a characteristic of some birthmarks and some skin diseases. Currently, correction of these conditions often encompasses painful grafting to replace burned skin, or surgery, e.g., laser surgery or excision of the area of unwanted coloration.

Sometimes, it is also desirable to lighten hair, wool or fur. For example, some people lighten, or "bleach" facial or scalp hair for cosmetic purposes. Some currently available methods for bleaching hair typically use harsh chemicals that can irritate sensitive skin surrounding the hair and damage the hair shaft, sometimes to the point of breakage. Furthermore, only the top of the hair shaft is affected by such treatment, leaving dark roots at, and below, the skin surface. These dark roots eventually grow out, necessitating repeated applications of these bleaching chemicals.

It would be advantageous to have available methods of decreasing, or suppressing, pigmentation in skin, hair, wool or fur without the need for surgical procedures or harsh chemicals.

SUMMARY OF THE INVENTION

The present invention is based on Applicants' discovery that activation of tyrosinase, the rate-limiting enzyme in melanogenesis, results from the protein kinase C-beta (also referred to herein as PKC-β) -mediated phosphorylation of serine and threonine residues of the cytoplasmic domain of tyrosinase.

Tyrosinase is found exclusively in melanocytes (pigment cells). These cells are located in the basal layer of the epidermis and in the hair bulb. Melanin pigment is deposited in melanocyte-specific organelles called melanosomes that are then transferred from the melanocyte to surrounding keratinocytes so that the pigment is widely dispersed through the epidermis (outer layer) of the skin or the hair shaft. The color (pigmentation) of vertebrate skin, hair, wool and fur is determined largely by its melanin pigment content.

Tyrosinase, a copper-binding transmembrane glycoprotein localized in the melanosome, is the principal and rate limiting enzyme in melanin synthesis by virtue of its ability to catalyze tyrosine hydroxylation and subsequent oxidation, the first two reactions in the biosynthetic sequence. Transfection experiments have established that tyrosinase alone enables otherwise non-melanogenic cells to produce melanin pigment (Bouchard, B, *et al.*, J. Exp. Med., 169:2029-2042 (1989)); and cloning of the human and murine tyrosinase genes has permitted mapping of numerous mutations responsible for albinism, a heritable loss of ability to pigment (King, R.A., *et al.*, PIGMENTATION AND PIGMENTARY DISORDERS, Levine, N., (ed.), CRC Press, Boca Raton. FL, pp. 297-336 (1993)).

Several other enzymes are known to participate in melanin biosynthesis. These include Tyrosinase Related Proteins 1 and 2 (TRP 1 and TRP 2) (Cohen, T., *et al.*, Nucleic Acids Res., 18:2807-2808 (1990); Jackson, I.J., *et*

al., EMBO J., 11:527-535 (1992)). As their names imply, the TRPs are structurally related to tyrosinase. In particular, the genes are homologous in the copper binding site and cysteine-rich domains, areas that are important for their structure and function (Hearing, V.J. and King, R.A., PIGMENTATION AND PIGMENTARY DISORDERS, Levine, N., (ed.), CRC Press, Boca Raton. FL, pp. 3-32 (1993)). The specific function of the TRPs are not known. However, recent data suggest that tyrosinase, TRP 1 and TRP 2 interact *in vivo* to form a complex, and that within this complex tyrosinase activity is diminished, suggesting that the TRPs may act as inhibitors of tyrosinase activity (Orlow, S.J., *et al.*, J. Invest. Dermatol., 103:196-201 (1994)).

In particular, Applicants have identified the specific serine residues in the cytoplasmic domain of tyrosinase that are phosphorylated by PKC-β. As a result of Applicants' discovery, methods are provided to modulate the activation of tyrosinase in vertebrate melanocytes. Modulate, as defined herein, means to alter the activation of tyrosinase either by preventing or inhibiting (decreasing) activation, or by enhancing or sustaining activation. For example, the activation of tyrosinase can be modulated by substantially decreasing, or completely blocking the PKC-β-mediated phosphorylation of tyrosinase, resulting in a decrease in melanogenesis. Conversely, tyrosinase activation can be modulated by enhancing activation, e.g., by facilitating the phosphorylation of tyrosinase, or sustaining activation, e.g., by preventing dephosphorylation of tyrosinase, resulting in an increase in melanogenesis.

Also provided are methods of altering pigmentation in vertebrate skin, hair, wool or fur as a result of modulating the activation of tyrosinase in melanocytes contained in the epidermis and in hair, wool and fur bulbs.

As used herein, the term epidermal melanocytes refers to melanocytes contained in the skin, and in the bulbs, or follicles of hair, wool and fur. The alteration of pigmentation, as used herein, means that pigmentation in epidermal melanocytes is either increased as a result of activation of tyrosinase which results in an increase in melanogenesis, or, alternatively, that pigmentation is decreased as a result of the inhibition of activation of tyrosinase, resulting in the decrease of melanogenesis.

One embodiment of the present invention relates to methods of preventing, or inhibiting, the activation of tyrosinase in vertebrate epidermal melanocytes. Tyrosinase is a monomeric protein with an inner domain, short transmembrane domain and a cytoplasmic domain. The cytoplasmic domain of tyrosinase contains serine residues. These serine amino acid residues are likely substrates for phosphorylation by PKC-β. As described herein, Applicants have demonstrated that serines at positions 505 and 509 of the tyosinase amino acid sequence (Shibahara, S., *et al.*, Tohoku J. Exp. Med., 156:403-411 (1988)) are sites of PKC-β-mediated phosphorylation of tyrosinase. Inhibiting the phosphorylation of serine 505 and/or 509 prevents the activation of tyrosinase.

Inhibition of the PKC-β-mediated phosphorylation of tyrosinase prevents activation of tyrosinase in epidermal melanocytes which results in a decrease in the production of melanin pigment in melanocytes. Thus, another embodiment of the present invention relates to decreasing, or completely suppressing pigmentation in vertebrate skin, hair, wool or fur.

Conversely, enhancing or sustaining tyrosinase activation by, e.g., preventing dephosphorylation of the activated tyrosinase, sustains, or prolongs melanogenesis in epidermal melanocytes, resulting in increased pigmentation of vertebrate skin, hair, wool or fur.

In another embodiment of the present invention, methods are provided to identify a substance which decreases, or completely suppresses, pigmentation in vertebrate epidermal melanocytes. Substances, for example, peptides, that specifically interfere with the interaction, or association, of PKC-β and tyrosinase mimic the sequence/structure of tyrosinase phosphorylation sites (e.g., peptide mimics), and bind to PKC-β, thereby preventing PKC-β from phosphorylating tyrosinase, and thus, preventing tyrosinase activation.

Conversely, methods are also provided to identify a substance which increases pigmentation in vertebrate epidermal melanocytes. Substances, for example, peptides, that specifically interfere with the phosphatases involved with the dephosphorylation of tyrosinase prevent tyrosinase deactivation. Substances identified by the methods described herein are also encompassed by the present invention.

As a result of Applicants' discovery of the specific sites of phosphorylation on tyrosinase, the rate-limiting enzyme in melanogenesis, methods are now available to modulate tyrosinase activation and pigmentation in vertebrate epidermal melanocytes. Methods are now available to increase pigmentation of skin, hair, wool and fur. Specifically, methods are now available to decrease, or suppress, (partially or completely) pigmentation of the skin, hair, wool and fur without surgery or harsh chemicals.

BRIEF DESCRIPTION OF THE FIGURE

The Figure is a graphic representation of experimental results showing that a synthetic peptide constructed to mimic the phosphorylation site of human tyrosinase inhibits tyrosinase activity in cultured human melanocytes.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on Applicants' finding that activation of tyrosinase, the rate-limiting enzyme in melanogenesis, results from the protein kinase C-beta (also referred to herein as PKC-β) -mediated phosphorylation of serine and threonine residues of the cytoplasmic domain of tyrosinase. Tyrosinase is a transmembrane protein localized to the melanosomes contained in melanocytes. Nucleotide sequences of cDNA clones for human tyrosinase have been reported in Shibahara, S., *et al.* Tohoku J. Exp. Med., 156:403-414 (1988); Chinatamaneni, C.D., *et al.* Proc. Natl. Acad. Sci. U.S.A., 88:5272-5276 (1991) and Kown, B.S. *et al.* Proc. Natl. Acad. Sci. USA, 84:7473-7477 (1987), the teachings of which are incorporated herein by reference. The putative human tyrosinase is composed approximately of 511 amino acids (Shibahara, S., *et al.* Tohoku J. Exp. Med., 156:403-414 (1988)). The cytoplasmic domain comprises two serine residues at the 505 and 509 positions of the amino acid sequence of Shibauhaer *et al.* and Chinatamanei, C.D., *et al.*

Tyrosinase is activated by the beta isoform of protein kinase C (PKC-beta). In the absence of PKC-beta, no melanin pigment is formed (Park, H-Y, *et al.*, J. Biol. Chem., 268:11742-11749 (1993)). Conversely, activation of PKC-beta above basal levels increases pigmentation in cultured human melanocytes, murine melanoma cells and in guinea pig skin. PKC-beta (PKC-β) is a serine/threonine kinase and activates proteins by phosphorylation of these amino acid residues. Park, H-Y. and Gilchrest, B.A., J. Dermatol. Sci., 6:185-193 (1993)). Full length tyrosinase shows incorporation of 22p phosphase, but the inner domain alone does not, suggesting that PKC-B phosphorylates only the cytoplasmic domain (Park, H-Y., *et al.*, J. Invest Dermatol., 104:585 Abstract 166 (1995)).

As described herein, Applicants have now demonstrated that activation of PKC-β in melanocytes leads to phosphorylation of tyrosinase, and specifically to phosphorylation of serine residues at positions 505 and 509 in the cytoplasmic domain of tyrosinase. Applicants have further demonstrated that removal of the cytoplasmic domain of tyrosinase by proteolysis prevents its phosphorylation by PKC-β. Thus, Applicants have demonstrated that preventing PKC-β-mediated phosphorylation of tyrosinase prevents activation of the rate limiting enzyme in melanogenesis and results in decreased, or completely suppressed, pigmentation in the target tissue, for example, vertebrate skin, hair, wool or fur.

Molecules or substances that specifically interfere with, or block, the interaction, or association, of PKC-β and tyrosinase can specifically inhibit, or substantially decrease the activation of tyrosinase. For example, molecules that specifically interfere with the phosphorylation of either serine residue 505 and 509, or both, can prevent tyrosinase activation. If tyrosinase is not activated, melanogenesis is significantly inhibited, resulting in decreased pigmentation, or complete suppression of pigmentation, in epidermal melanocytes.

Molecules such as proteins, peptides, antibodies and antibody fragments can interfere with the interaction between PKC-β and tyrosinase. Organic and inorganic molecules can also interfere with this interaction. Such molecules can be naturally occurring, and purified, or isolated from their natural environment, using techniques well-known to those of skill in the art. Such molecules can also be synthesized by chemical means, or recombinantly produced, also using techniques well-known to those of skill in the art.

As used herein, specific interference of the interaction between PKC-β and tyrosinase refers to the

prevention, or blocking, of PKC-β-mediated phosphorylation of tyrosinase. The blocking can be complete blocking, or partial blocking, which results in the complete inhibition, or substantial reduction of melanogenesis in epidermal melanocytes. Complete inhibition of, or substantial reduction of melanogenesis in epidermal melanocytes results in decreased pigmentation, or completely suppressed pigmentation, in skin, hair, wool or fur in vertebrates.

Specifically encompassed by the present invention are peptides, or peptide fragments that mimic the sites of interaction between PKC-β and tyrosinase. These "peptide mimics" mimic the sites of PKC-β-mediated phosphorylation of tyrosinase, i.e., amino acid residues that comprise the substrate sequences of tyrosinase for PKC-β-mediated phosphorylation. The substrate sequences of tyrosinase mimics typically include a serine or threonine residue. The amino acid sequences of tyrosinase mimics include, for example, the serine residue 505 and 509, and their respective surrounding amino acid residues. These tyrosinase peptide mimics bind directly to PKC-β in a manner similar to the binding of PKC-β to tyrosinase, thereby preventing PKC-β from binding to tyrosinase and thus, preventing, reducing or completely eliminating the activation of tyrosinase and subsequent melanogenesis.

The tyrosinase mimics used in the methods described herein can be, e.g., proteins, peptides (comprised of natural and non-natural amino acids) or can be peptide analogs (comprised of peptide and non-peptide portions). Such peptide mimics can be constructed with D-isomers rather than the native L-isomers of the amino acids, to increase their resistance to proteolytic degradation within living cells. All tyrosinase mimics used in these methods have specific characteristics pertaining to biological activity. These characteristics include the ability of

these mimics to bind to PKC-β and the retention of a biologically active conformation.

The tyrosinase peptide mimics used in the methods described herein include at least five amino acid residues, and generally have a sequence in the range of about ten to about thirty amino acid residues, typically having about twenty residues. However, longer mimics can be used (e.g., the length of the entire cytoplasmic domain, or up to about 60-65 amino acid residues) if they have the desired characteristics described above. Typically, one of the residues of tyrosinase peptide mimics is a serine or threonine. For example, tyrosinase mimics such as SEQ ID NO: 1 and SEQ ID NO.: 4 are especially useful in the methods described herein.

Molecules that mimic the active sites of PKC-β can also be used in the methods described herein. Such a PKC-β mimic molecule would bind to the tyrosinase substrate sequence, but would not activate tyrosinase. However, the PKC-β mimic binding to the substrate prevents PKC-β binding to tyrosinase. Thus, the PKC-β mimic would also block the PKC-β-mediated phosphorylation of tyrosinase as a competitive antagonist, inhibiting the activation of tyrosinase and decreasing pigmentation. Such PKC-β mimics can comprise, for example, proteins, peptides, organic or inorganic molecules.

Tyrosinase mimics and PKC-β mimics can be rationally designed and synthetically produced by methods well-known to those of skill in the art, for example, as described in Jameson, B.A., *et al.*, Nature, 368:744-746 (1994). Candidate tyrosinase mimics and PKC-β mimics can be identified and screened for biological activity (i.e., the ability to block the interaction of PKC-β with tyrosinase) using *in-vitro* assays well-known to those of skill in the art.

For example, as described herein, one method comprises culturing melanocytes containing tyrosinase in the presence of a radioactive label such as ^{32}P-orthophosphate. The melanocytes can be obtained from skin biopsies, neonatal foreskins or melanoma cell lines (see, e.g., Park, H-Y. *et al.*, J. Biol. Chem., 268:11742-11749 (1993)). The melanocytes are contacted with a phorbol ester such as tetraphorbol acetate (TPA) to activate PKC-β. Other suitable PKC-β activators known to those of skill in the art can also be used. Simultaneously, or subsequentially, the cultured melanocytes are contacted with the substance to be tested under conditions suitable for PKC-β-mediated phosphorylation of tyrosinase. Tyrosinase-containing melanosomes are then purified from the TPA-treated melanocytes and tyrosinase is isolated from the melanosomes. Isolation can be accomplished by standard laboratory techniques. In particular, isolation by immunoprecipitation with an antibody specific for tyrosinase is encompassed by the present method. The antibody can be polyclonal or monoclonal. (See e.g., Jimenez, M. *et al.*, J. Biol. Chem., 266:147-1156 (1991); Bouchard, B. *et al.*, J. Invest. Dermatol., 102:291-295 (1994); or EPO 679,660 A1 02/11/95).

Immunoprecipitation assays can be performed as described in e.g., Park, H-Y. *et al.*, J. Biol. Chem., 268:11742-11749 (1993). The amount of ^{32}P-orthophosphate incorporated in the immunoprecipitated tyrosinase is determined using standard laboratory techniques, and the amount of ^{32}P-orthophosphate incorporated into tyrosinase isolated from melanocytes cultured in the presence of a test substance is compared with the amount of ^{32}P-orthophosphate incorporated into tyrosinase isolated from melanocytes cultured in the absence of a test substance. A decreased amount of ^{32}P-orthophosphate incorporation into tyrosinase is an indication that the test substance

prevents phosphorylation of (i.e., activation of) tyrosinase. As the test substance prevents activation of tyrosinase, and tyrosinase is essential for melanogenesis and pigmentation, pigmentation in vertebrate epidermal melanocytes is completely inhibited or substantially decreased.

Alternatively, tyrosinase activity can be directly measured using other well-known laboratory techniques. For example, Pomerantz, S.H. describes an assay to measure tyrosinase activity (J. Biol. Chem., 241:161-168 (1966), the teachings of which are incorporated by reference). In brief, 5 x 10^4 cells are briefly sonicated in 80 mM PO_4^{3-} (pH 6.8) containing 1% Titon X-100, and tyrosinase is extracted for 60 minutes at 4°C. 10-50 µg of cellular protein are incubated with 250 nM L-tyrosine, 25 nM L-deoxyphenylalanine, 12.5 µg of chloramphenicol, and 5 µCi of L-[3,5-^{3}H]tyrosine for 30-60 minutes at 37°c. The reaction is stopped by addition of 500 µl of 10% trichloroacetic acid containing 0.2% BSA. Trichloroacetic acid-soluble material is reacted with Norit A, and released ^3H_2O is measured using a scintillation counter. The activity is expressed as counts/minute ^3H_2O released/µg protein/h minus the nonspecific incorporation of radioactivity, determined by using lysate boiled for 30 minutes (background).

Additionally, the melanin content of melanocytes can be measured directly, as described, for example, in Gordon, P.R. and Gilchrest, B.A., J. Invest. Dermatol., 93:700-702 (1989), the teachings of which are also incorporated herein by reference. Briefly, human melanoma cells are cultured under standard laboratory conditions. 1 X 10^5 cells are routinely used to measure melanin content. Cells are spun at 2,500 rpm for 15 minutes and the resulting pellet is dissolved in 0.5 ml of 1 N NaOH. Melanin concentration is

calculated by OD_{475} and comparison with a standard curve of synthetic melanin.

Tyrosinase mimics and PKC-β mimics that exhibit activity *in vitro* can be further tested *in-vivo*, for example, by topical application to guinea pig skin or hair, as described in Eller, M. *et al.*, Nature, 372:413-414 (1994) or Allen *et al.*, J. Invest. Dermatol., (1995), the teachings of which are incorporated herein by reference.

Conversely, Applicants' discovery regarding PKC-β activation of tyrosinase, the rate limiting enzyme in melanogenesis, provides methods of increasing synthesis of melanin and hence darkening of skin, hair, wool or fur. Methods are also provided herein to enhance, or to maintain, the steady state level of phosphorylation of tyrosinase by PKC-β as a result of blocking the dephosphorylation of serine and threonine residues of the cytoplasmic domain of tyrosinase by providing a false substrate for the relevant phosphorylase in the cells.

Specifically, the rate of melanin synthesis in melanocytes is known to be determined by the state of activation of tyrosinase. This activation state is a dynamic equilibrium between activation (phosphorylation) of the enzyme and deactivation (dephosphorylation) of the enzyme that is associated with the intracellular melanosomes. Phosphorylation is mediated by PKC-β and dephosphorylation is mediated by one or more phosphatases. It is reasonable to assume that different phosphatases are responsible for dephosphorylating different PKC-β substrates, as would be required in a situation in which multiple PKC substrates must be regulated independently for normal cell function.

A peptide sequence specific for the catalytic domain of the phosphatase responsible for dephosphorylation of PKC-β-activated tyrosinase can be constructed and delivered to melanocytes in the skin, hair, wool or fur or any

additional site where increased melanogenesis is desired. This "false substrate" for the phosphatase competes with the phosphorylated site on tyrosinase for its active site and thus reduces the availability of the phosphatase to the physiologic substrate, tyrosinase. This method can be used when increased melanin content is desired, e.g., in areas of post-inflammatory hypopigmentation of the skin as commonly occurs in patients with low grade eczematous dermatitis, or to darken human hair shade, or the color of animal fur or wool.

Thus, providing substances to cells that modulate the activation of tyrosinase can modulate the synthesis of melanin in epidermal melanocytes. Specifically, providing tyrosinase peptide mimics to cells or tissues can result in competitive interaction of the added peptide with PKC-β, decreasing the availability of PKC-β to interact with its normal intracellular substrate, in this instance the cytoplasmic domain of tyrosinase contained within the melanocyte. Such a substrate sequence can interact specifically or preferentially with PKC-β rather than with other PKC isomers because the other PKC isomers do not activate tyrosinase protein. Although PKC isomers are found ubiquitously in cells and tissues, and are known to mediate a wide variety of critical cellular functions, a substrate sequence specific for PKC-β acts preferentially on the melanogenic pathway in melanocytes because PKC-β is minimally expressed in keratinocytes or fibroblasts, the other major cell types in the skin (Park, H-Y. *et al.*, Clin. Res., 39:148A (1991)).

Furthermore, it is known that human melanoma cell lines completely lacking PKC-β are indistinguishable from parental lines expressing this PKC isoform, except for the fact that they lack melanin pigment (Park, H.E. *et al.*, J. Biol. Chem., 268(16):11742-11749 (1993)). This strongly suggests that PKC-β does not serve any other major function

in melanocyte/melanoma cells, aside from its role in melanogenesis.

The methods of the present invention can be used to alter pigmentation in vertebrate melanocytes via modulation of the activation of tyrosinase. Specifically, the methods described herein can be used to decrease, or completely suppress, pigmentation in vertebrate skin, hair, wool or fur. These methods comprise contacting, epidermal cells in such a manner that the substance enter the cell, including basal layer melanocytes, (e.g., introducing into, delivering to, or administering to) with an effective amount of a substance which of decreases, or suppresses, pigmentation by decreasing or inhibiting the activation of tyrosinase in melanocytes. For example, the substance can be contained in a physologically compatible composition which is topically applied to the skin, or skin surrounding the hair, wool or fur bulbs.

Conversely, the methods of the present invention can also be used to increase pigmentation in vertebrate skin, hair, wool or fur. These methods comprise contacting, or delivering to, epidermal melanocytes (melanocytes located in the skin or hair, wool or fur bulbs) with an effective amount of a substance which increases pigmentation by enhancing or sustaining the activation of melanocytes in vertebrates.

An effective amount of such an identified substance is an amount effective to modulate (e.g., substantially reduce, or completely inhibit, or substantially enhance or sustain) PKC-β-mediated phosphorylation of tyrosinase in epidermal melanocytes. The modulation of tyrosinase phosphorylation in melanocytes can be evaluated using the methods described herein.

Various delivery systems suitable for use in the present invention are known to those of skill in the art and can be used to administer effective amounts of

substances, such as tyrosinase peptide mimics, to inhibit activation of tyrosinase in melanocytes. For example, protein encapsulation in liposomes, microparticles, or microcapsules; expression by recombinant cells, receptor-mediated endocytosis, construction of a naturally-occurring or pseudo-ligand encoding nucleic acid as part of a retroviral or other vector can be used.

In one embodiment, a liposome preparation can be used. The liposome preparation can be comprised of any liposome which penetrates the stratum corneum and fuses with the cell membrane, resulting in delivery of the contents of the liposome into the cell. Liposomes can be prepared by methods well-known to those of skill in the art. For example, liposomes such as those described in U.S. Patent No. 5,077,211; No. 4,621,023; No. 4,880,635 or No. 5,147,652 can be used. See also, Yarosh, D., *et al.*, J. Invest. Dermatol., 103(4):461-468 (1994) or Caplen, N.J., *et al.*, Nature Med., 1(1):39-46 (1995).

The liposomes can specifically target the appropriate cells (e.g., epidermal melanocytes). For example, a membrane marker preferentially expressed on melanocytes, such as melanocyte stimulating hormone (MSH) receptor, can be incorporated into a liposome containing a peptide mimic that prevents the activation of tyrosinase. Liposomes can also specifically target and deliver substances to the hair follicles, as described below in Li, L. and Hoffman, R.M., *et al.*, the teachings of which are incorporated herein by reference. Such a liposome delivery system can also be used to deliver substances to wool and fur bulbs.

For example, a peptide mimic, or a DNA construct encoding a peptide mimic, can be encapsulated into a liposome by techniques well known to those of skill in the art. The DNA construct will comprise the DNA sequence encoding the peptide and other nucleic acid sequences necessary for the expression of the peptide in vertebrate

cells. (See, for example, Li, L. and Hoffman, R.M., Natured Med., 1:705-706 (1995) or Yarosh, D. et al., J. Invest.Dermatol., 103:461-468 (1994)). The liposome-DNA construct, or liposome-peptide composition (containing the peptide mimic) can be administered to the vertebrate, for example, by topical application to the skin, hair, wool or fur, or to the skin surrounding the hair, wool or fur bulb. The liposome-DNA construct, or liposome-peptide composition contacts the melanocytes, with the result that the contents of the liposome (DNA or peptide) are introduced into the melanocytes in which the peptide mimic is expressed, or released, resulting in modulation of tyrosinase activation.

Substances used in the present methods can also be directly administered in a physiologically compatible carrier. For example, peptides of the size required to competitively inhibit PKC-β phosphorylation of tyrosinase are sufficiently small to permit their transepidermal delivery to melanocytes in the epidermis and hair, wool or fur bulb using existing technology. The peptide can be admixed in a topical carrier such as a gel, an ointment, a lotion, a cream, or a foam, or a shampoo and will include such carriers as water, glycerol, alcohol, propylene glycol, fatty alcohols, triglycerides, fatty acid esters, or mineral oils. Other possible topical carriers include, e.g., liquid petrolatum, isopropyl palmitate, polyethylene glycol, ethanol (95%), polyoxyethylene monolauriate (5%) in water, sodium lauryl sulfate (5%) in water. Other materials such as anti-oxidants, humectants, viscosity stabilizers, and similar agents may be added as necessary.

In addition, in certain instances, it is expected that the substances can be disposed within devices placed upon, in, or under the skin. Such devices include transdermal patches, implants, and injections which release the substances in such a manner as to contact the skin or hair follicle either by passive or active release mechanisms.

substances in such a manner as to contact the skin or hair follicle either by passive or active release mechanisms.

The delivery vehicle can also contain perfumes, colorants, stabilizers, sunscreens, or other ingredients. The substance can be applied, for example, topically to the epidermis at regular intervals, such as once or twice daily, in a suitable vehicle and at an effective concentration.

An effective amount of a mimic substance that modulates the activation of tyrosinase can be administered to a vertebrate, including a human, using any of the above-described methods. The actual preferred amounts of substance to be administered will vary according to the specific mimic being utilized, the particular compositions formulated, the mode of application, and the particular sites and vertebrate being treated. The concentration of the mimic effective to prevent activation of tyrosinase, in a vertebrate, such as a human, can be determined using known, conventional pharmacological protocols.

The present invention also encompasses methods of identifying a substance capable of altering pigmentation in vertebrate epidermal melanocytes, and the substances identified by these methods. These methods identify substances based on the effect the substance has on the protein kinase-C-β-mediated activation of tyrosinase in epidermal melanocytes.

For example, vertebrate epidermal melanocytes are grown in culture under conditions suitable for maintaining the growth and viability of the melanocytes. The substance to be tested (i.e., the test-substance) is then introduced into the culture and thus, into the cultured cells. The culture containing the test substance is maintained under conditions suitable for the substance to affect tyrosinase activity, e.g., to inhibit protein kinase-C-β-mediated phosphorylation of tyrosinase. Control cultures of

melanocytes are also maintained under similar conditions but without the presence of substance to be tested. After a suitable period of time, the melanocytes are removed, or isolated, from the culture, e.g., by centrifugation, and tyrosinase is isolated from the melanocytes, e.g., by immunoprecipitation with tyrosinase-specific antibody. Immunoprecipitation can also be accomplished using a specific antibody that recognizes a fragment of tyrosinase, e.g., the tyrosinase cytoplasmic domain.

Phosphorylation of tyrosinase is then evaluated. Evaluation of phosphorylated typically encompasses quantifying the amount of, or the magnitude of, phosphorylation that has occurred while the melanocytes were cultured with the test-substance. A standard method of quantification is determining the amount of radiolabeled phosphate incorporated into tyrosinase, for example, ^{32}P-orthophosphate. Phosphorylation of tyrosinase isolated from melanocytes cultured in the presence of test substance is then compared to the phosphorylation of tyrosinase isolated from melanocytes cultured without test substance. If the substance has the effect of inhibiting protein kinase C-β-mediated phosphorylation of tyrosinase, then the amount of phosphorylation of tyrosinase isolated from cultured melanocytes grown in the presence of the test substance will be less than the amount of phosphorylation of tyrosinase isolated from control melanocytes.

The present method can also be used to identify a substance that has the effect of increasing pigmentation in vertebrate epidermal melanocytes. Such substances would have the effect of enhancing the phosphorylation of or sustaining the phosphorylated state of tyrosinase. The steps of the method are similar to the above discussed method, except that the test substance possessed the desired characteristics of enhancing phosphorylation, the extent of phosphorylation of tyrosinase isolated from

isolated from melanocytes cultured without test substance. To determine if a test substance had the effect of sustaining or prolonging the state of phosphorylation of tyrosinase (thus, sustaining or prolonging the activation of tyrosinase), the melanocyte cultures can be maintained with or without test substance for prolonged periods of time prior to isolating melanocytes and evaluating the phosphorylation of tyrosinase isolated from the melanocytes.

Substances identified by these *in vitro* methods can be further tested *in vivo*, for example, in guinea pigs, as described in Eller, M.S. *et al.*, Nature, 372:413-414 (1944), the teachings of which are incorporated herein by reference. Substances that are effective *in vivo* can be used in the methods of altering pigmentation of vertebrate epidermal melanocytes as described above.

The following examples more specifically illustrate the invention and are not intended to be limiting in any way.

EXAMPLE 1: PROTEIN KINASE C-BETA PHOSPHORYLATES TYROSINASE

To examine if only PKC-β, but not other isoforms, phosphorylates tyrosinase in vivo, melanocytes which express PKC-β and non-pigmented-MM4 human melanoma cells that comparably express tyrosinase, but lack the expression of PKC-β, were preincubated with ^{32}P-orthophosphate for 90 minutes. (J. Invest. Derm., Vol. 100, No.4: Abst. #37 (April 1993). PKC was activated by treating cells with 10^{-7}M TPA, a well known activator of all PKC isoforms, for 30 minutes. Control cells received vehicle only. Subsequently, tyrosinase was immunoprecipitated using a polyclonal antibody against human tyrosinase and incorporation of ^{32}P-orthophosphate into tyrosinase was visualized by autoradiography. Tyrosinase was only phosphorylated in TPA-treated melanocytes that express PKC-

visualized by autoradiography. Tyrosinase was only phosphorylated in TPA-treated melanocytes that express PKC-β, suggesting that only PKC-β, but not other PKC isoforms, can phosphorylate tyrosinase in vivo.

EXAMPLE 2: TYROSINASE ACTIVITY IS UP-REGULATED BY PHOSPHORYLATION

To determine whether the activity of tyrosinase can be up-regulated by phosphorylation, purified mushroom tyrosinase was pre-incubated with purified activated PKC-β (Park, H-Y. *et al.*, J. Invest Dermatol., 100:607 (1993) and Park, H-Y. *et al.*, J. Biol. Chem., 268:11742-11749 (1993)). Subsequently tyrosinase activity was visualized by separating tyrosinase and PKC-β in a non-denaturing 7.5% acrylamide gel-electrophoresis, followed by reaction with L-dopa. A brown colored band (melanin) appears where tyrosinase migrates, its intensity corresponding to enzymatic activity. Purified tyrosinase alone showed some activity and the activity increased more than 10 fold when tyrosinase was pre-incubated with purified activated PKC-β. This increase is not due to a possible interaction between PKC-β and L-dopa since PKC-β alone did not react with L-dopa. These data clearly show that direct phosphorylation of tyrosinase by PKC-β leads to activation of the enzyme.

EXAMPLE 3: PROTEIN KINASE C-BETA PHOSPHORYLATES THE CYTOPLASMIC DOMAIN OF TYROSINASE

The amino acid sequence of human tyrosinase has been previously reported. Tyrosinase has two serine residues at positions 505 and 509 in cytoplasmic domain (Shibahara, S., *et al.* Tohoku J. Exp. Med., 156:403-414 (1988). Approximately 90% of the tyrosinase protein is inside melanosomes, membrane-bound organelles within melanocytes in which melanin pigment is synthesized and deposited. PKC-β normally resides in the cytoplasm. To examine if

only the cytoplasmic domain of tyrosinase is phosphorylated by PKC, melanocyte cultures were preincubated with ^{32}P-orthophosphate, PKC was activated by treating with 10^{-7}M TPA for 60 minutes, and the melanosomes (which contain tyrosinase) were purified using sucrose gradient centrifugation.

Purified melanosomes were divided into two groups: one group remained untreated as a control and the other group was treated with 0.25% trypsin for 60 minutes at 37°C to release the cytoplasmic domain. Subsequently, trypsin-treated and untreated tyrosinase (full-length) was extracted from melanosomes by incubating in 0.1% Triton X-100 for 60 minutes and treated or untreated tyrosinase was immunoprecipitated using an polyclonal antibody specifically reacting against the inner (intra-melanosomal) domain or a polyclonal antibody against the full length tyrosinase respectively.

^{32}P-orthophosphate was incorporated only into untreated or full length tyrosinase (Park, H-Y., *et al.*, J. Invest. Dermatol., 1041, 585 Abstract 186 (April 1995)). Trypsin-treated tyrosinase, lacking the cytoplasmic domain, failed to show incorporation of ^{32}P-orthophosphate, demonstrating that only the cytoplasmic domain is phosphorylated by PKC. In a parallel experiment, melanocyte cultures were processed as above except that phosphorylation of tyrosinase was done in the absence of radiolabeled phosphate. Immunoblot analysis with untreated and trypsin-treated tyrosinase confirmed that a comparable amount of tyrosinase was immunoprecipitated using two different antibodies.

EXAMPLE 4: PROTEIN KINASE C-BETA PHOSPHORYLATES SERINE AND THREONINE RESIDUES

To examine if both serine and threonine residues are phosphorylated by PKC, a known serine/threonine kinase,

tyrosinase was phosphorylated *in vivo* by preincubation of melanocytes with ^{32}P-orthophosphate, followed by activation of PKC with TPA, as described in Example 1. Subsequently, tyrosinase was immunoprecipitated, electroeluted from the gel and subjected to a full hydrolysis. Radiolabeled amino acids were separated using two-dimensional thin-layer chromatography using standard techniques, and mapped against unlabeled and phosphorylated serine, threonine and tyrosine standards.

Results showed that >90% of the radiolabeled phosphate was associated with serine. Radiolabeled phosphate associated with tyrosine was not detected. These data further demonstrate that tyrosinase is phosphorylated through the PKC-dependent pathway and that serine residues are preferentially phosphorylated.

EXAMPLE 5: SERINES 505 AND 509 ARE PHOSPHORYLATED

To identify the exact serine or threonine residues predominantly phosphorylated by PKC-β, tyrosinase was labeled with ^{32}P-orthophosphate by incubating melanocytes with radiolabeled phosphate and activating PKC. Subsequently melanosomes were purified and full length tyrosinase was immunoprecipitated. Tyrosinase was then treated with trypsin, then with thermalysin. Since only the cytoplasmic domain is phosphorylated, digestion of full length tyrosinase should generate only phosphorylated cytoplasmic domain.

Based on the amino acid sequence of human tyrosinase by Sibahara, it was predicted that three fragments will be generated when the cytoplasmic domain of human tyrosinase is digested with trypsin. Synthetic peptides were made with or without a phosphate group on the serine residues. In three independent experiments, all of the readiolabled phosphate was mapped to synthetic peptide 3, indicating that both serines residues are phosphorylated by PKC-β.

Cytoplasmic Domain of Tyrosinase

Gln-Leu-Pro-Glu-Glu-Lys-Gln-Pro-Leu-Leu-Met-Glu-Lys-Glu-Tyr-His-$(Ser)_{505}$-Leu-Tyr-Gln-$(Ser)_{509}$-His-Leu (SEQ ID NO: 1)

↓ Trypsin

Fragment 1: Gln-Leu-Pro-Glu-Glu-Lys (SEQ ID NO: 2)
Fragment 2: Gln-Pro-Leu-Leu-Met-Glu-Lys (SEQ ID NO: 3)
Fragment 3: Glu-Asp-Tyr-His-$(Ser)_{505}$-Leu-Tyr-Gln-$(Ser)_{509}$-His-Leu (SEQ ID NO: 4)

Synthetic Peptides:

Peptide 1:: Glu-Asp-Tyr-His-$_p(Ser)_{505}$-Leu-Tyr-Gln-$(Ser)_{509}$-His-Leu (SEQ ID NO: 4)
Peptide 2:: Glu-Asp-Tyr-His-$(Ser)_{505}$-Leu-Tyr-Gln-$_p(Ser)_{509}$-His-Leu (SEQ ID NO: 4)
Peptide 3:: Glu-Asp-Tyr-His-$_p(Ser)_{505}$-Leu-Tyr-Gln-$_p(Ser)_{509}$-His-Leu (SEQ ID NO: 4)

EXAMPLE 6: SYNTHETIC PEPTIDE INHIBITS TYROSINASI ACTIVITY

Paired cultures of human melanocytes were either untreated or treated with 5, 10 or 20 ug/dish of synthetic peptide whose sequence is identical to the part of human tyrosinase containing serines residues at amino acid positions 505 and 509. The specific sequence of the synthetic peptide is Glu-Asp-Tyr-His-$(Ser)_{505}$-Leu-Tyr-Gln-$(Ser)_{509}$-His-Leu (SEQ ID NO: 4). The synthetic peptide was pretreated with 10 ul of Lipofectamine to enhance delivery into the cells. Control designates no treatment and 0 designates LIpofectamine alone. Melanocytes were exposed to Lipofectamine treated synthetic peptides for 22 hours, harvested and tyrosinase activity was determined.

TABLE I					
	Tyr Act/ ug Protein	CPM 1	CPM 2	CPM neg1	CPM neg2
Control	14679.2	118490	119159	50443	40414
0	14807.4	126917	119698	50248	48203
5	17152.6	148889	119059	47579	48843
10	9536.9	96566	93442	46135	48504
20	7811.3	90265	84051	46312	49891

As shown in Table I and the Figure, tyrosinase activity in cultured human melanocytes is inhibited by more than 50% in cultures treated with 20 ug/dish, equivalent to 2 ug/ml, of the synthetic peptide constructed to mimic the phosphorylation site on human tyrosinase. Approximately 40% inhibition was observed at the next lower dose of 1 ug/ml

EXAMPLE 7: PROTEIN KINASE C-BETA INTERACTION WITH TRP 1 AND TRP 2

To investigate whether PKC regulates other melanogenic proteins such as tyrosinase related proteins (TPR 1 and TPR 2), melanocytes were treated with 10^{-7}M TPA for 2 weeks, a condition known to deplete PKC, and TRP 1 and TRP 2 protein levels were determined using immunoblot analysis with specific antibodies. PKC depletion had no effect on TPR 1, but the level of the glycosylated mature form of TRP 2 (80 kd) was reduced by 50-70%. The 65 kd non-glycosylated TRP 2 precursor was unaffected by depletion of PKC. To confirm that indeed only the glycosylated TRP 2 is affected, melanocytes were treated with TPA for 2 weeks and labeled with ^{3}H-glycosamine. TRP 1 and TRP 2 were immunoprecipitated and incorporation of ^{3}H-glycosamine into these proteins was examined. PKC depletion did not affect

either the glycosylated or non-glycosylated form of TPR 1, but ^{3}H-glycosamine incorporation into TRP 2 was reduced by >50%. Together these results suggest that PKC regulates melanogenesis by preferentially phosphorylating serine residues on the cytoplasmic domain of tyrosinase and by regulating the level of mature TRP 2.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

What is claimed is:

1. A method of modulating the activation of tyrosinase in vertebrate epidermal melanocytes comprising modulating the protein kinase C-beta-mediated phosphorylation of a serine or a threonine residue of the tyrosinase cytoplasmic domain.

2. A method of preventing the activation of tyrosinase in vertebrate epidermal melanocytes comprising inhibiting the protein kinase C-beta-mediated phosphorylation of a serine or a threonine residue of the tyrosinase cytoplasmic domain.

3. A method according to Claims 1 or 2 wherein the phosphorylation of serine residues 505, 509 or both 505 and 509 are inhibited.

4. A method of preventing the activation of tyrosinase in vertebrate epidermal melanocytes comprising introducing into the melanocytes a peptide that specifically interferes with the protein kinase C-beta-mediated phosphorylation of serine/threonine residues in the cytoplasmic domain of tyrosinase.

5. A method according to Claim 4 wherein the phosphorylation of serines residue 505, 509 or both 505 and 509 are inhibited.

6. A method according to Claim 4 wherein the peptide comprises an amino acid sequence homologous to the amino acid sequence comprising the site of the

cytoplasmic domain of tyrosinase where protein kinase C-beta-mediated phosphorylation occurs.

7. A method according to Claim 6 wherein the peptide comprises from about 5 to about 30 amino acid residues and at least one of the residues is a serine or threonine.

8. A method according to Claim 7 wherein the peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO.: 4.

9. A method of altering pigmentation in vertebrate skin, hair, wool or fur comprising modulating the protein kinase C-beta-mediated phosphorylation of a serine or a threonine residue in the cytoplasmic domain of tyrosinase contained in epidermal melanocytes.

10. A method according to Claim 9 wherein pigmentation in vertebrate skin, hair, wool or fur is decreased comprising inhibiting the protein kinase C-beta-mediated phosphorylation of a serine or a threonine residue in the cytoplasmic domain of tyrosinase contained in epidermal melanocytes.

11. A method according to Claim 10 wherein the phosphorylation of serines residue 505, 509 or both 505 and 509 are inhibited.

12. A method according to Claim 10 wherein a peptide that specifically interferes with the protein kinase C-beta-mediated phosphorylation of a serine or a threonine residue in the cytoplasmic domain of tyrosinase is introduced into epidermal melanocytes.

13. A method according to Claim 12 wherein the peptide comprises an amino acid sequence homologous to the amino acid sequence comprising the site of the cytoplasmic domain of tyrosinase where protein kinase C-beta-mediated phosphorylation occurs.

14. A method according to Claim 13 wherein the peptide comprises from about 5 to about 30 amino acid residues and at least one of the residues is a serine or threonine.

15. A method according to Claim 14 wherein the peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO.: 4.

16. A method according to Claim 12 wherein the peptide is introduced into the epidermal melanocytes by topical administration of a pharmaceutical composition containing the peptide.

17. A method of decreasing pigmentation in vertebrate skin, hair, wool or fur comprising inhibiting the phosphorylation of tyrosinase in vertebrate epidermal melanocytes contained in skin or hair, wool or fur bulbs comprising topically applying to the skin or hair, wool or fur bulbs of the vertebrate, a DNA construct encapsulated into a liposome wherein the DNA construct encodes a peptide comprising an amino acid sequence homologous to the amino acid sequence comprising the site of the cytoplasmic domain of tyrosinase where protein kinase C-beta-mediated phosphorylation occurs, whereby the DNA construct is introduced into the melanocytes and the peptide is expressed in the melanocytes, resulting in inhibition

of the phosphorylation of tyrosinase, thereby decreasing pigmentation.

18. A method of identifying a substance which alters pigmentation in vertebrate epidermal melanocytes, comprising evaluating the effect the substance has on the protein kinase-C-beta-mediated activation of tyrosinase in epidermal melanocytes, wherein if protein kinase C-beta-mediated activation of tyrosinase is altered, the pigmentation in vertebrate melanocytes is altered.

19. A method of identifying a substance which decreases pigmentation in vertebrate epidermal melanocytes comprising determining the effect the substance has on inhibiting the protein kinase-C-beta-mediated phosphorylation of tyrosinase in the melanocytes, comprising the steps of:

a) introducing into cultured vertebrate melanocytes a test substance in an amount sufficient to inhibit phosphorylation of tyrosinase if the substance possesses inhibiting properties;

b) isolating tyrosinase from the cultured melanocytes of step a) and evaluating phosphorylation of the isolated tyrosinase; and

c) comparing the phosphorylation of tyrosinase isolated from the cultured melanocytes of step a) with the phosphorylation of tyrosinase isolated from melanocytes cultured under similar conditions but in the absence of the test substance to determine the effect the substance has on the inhibition of protein kinase-C-beta-mediated phosphorylation of tyrosinase, whereby if the substance inhibits protein kinase-c-beta-

mediated phosphorylation of tyrosinase, pigmentation in melanocytes is decreased.

20. The method of Claim 19, wherein the melanocytes are cultured in the presence of ^{32}P-orthophosphate; in step b), the amount of ^{32}P-orthophosphate incorporated into the isolated tyrosinase is determined, and in step c), the amount of ^{32}P-orthophosphate incorporated into tyrosinase isolated from the cultured melanocytes of a) is compared with the amount of ^{32}P-orthophosphate incorporated into tyrosinase isolated from melanocytes cultured under similar conditions but in the absence of the test substance.

21. The method of Claim 19 wherein the isolation of tyrosinase from cultured melanocytes in step b) is by immunoprecipitation with an antibody specific for tyrosinase or a fragment thereof.

22. A substance that inhibits the protein kinase C-beta-mediated phosphorylation of tyrosinase and identified or identifiably by the method of Claim 19; and for example for use in therapy.

23. A method of identifying a substance which increases pigmentation in vertebrate epidermal melanocytes comprising determining the effect the substance has on enhancing the protein kinase-C-beta-mediated phosphorylation of tyrosinase in melanocytes, comprising the steps of :

a) introducing into cultured vertebrate melanocytes a test substance in an amount sufficient to enhance phosphorylation of tyrosinase if the substance possesses enhancing properties;

b) isolating tyrosinase from the cultured melanocytes of step a) and evaluating the phosphorylation of the isolated tyrosinase; and

c) comparing the phosphorylation of tyrosinase isolated from the cultured melanocytes of step a) with the phosphorylation of tyrosinase isolated from melanocytes cultured under similar conditions but in the absence of the test substance to determine the effect the substance has on the enhancement of protein kinase-C-beta-mediated phosphorylation of tyrosinase, whereby if the substance inhibits protein kinase-c-beta-mediated phosphorylation of tyrosinase, pigmentation in melanocytes is increased.

24. A substance that enhances the protein kinase C-beta-mediated phosphorylation of tyrosinase and identified or identifiably by the method of Claim 23; and for example for use in therapy.

25. Use of an agent which modulates the protein kinase C-beta-mediated phosphorylation of a serine or a thrsonine residue of the tyrosinase cytoplasmic domain contained in vertebrate epidermal melanocytes, for the manufacture of a medicament for modulating the activation of tyrosinase in vertebrate epidermal melanocytes.

26. Use of an agent which inhibits the protein kinase c-beta-mediated phosphorylation of a serine or a thrsonine residue of the tyrosinase cytoplasmic domain contained in vertebrate epidermal melanocytes, for the manufacture of a medicament for preventing the activation of tyrosinase in vertebrate epidermal melanocytes.

27. Use of a peptide that specifically interferes with the protein kinase C-beta-mediated phosphorylation of serine/threonine residues in the cytoplasmic domain of tyrosinase in vertebrate epidermal melanocytes, for the manufacture of a medicament for preventing the activation of tyrosinase in vertebrate epidermal melanocytes by introducing the peptide into the melanocytes.

28. Use according to Claim 27, wherein the medicament is for altering pigmentation in vertebrate skin, hair, wood or fur.

29. Use according to Claim 28, wherein the medicament is for decreasing pigmentation in vertebrate skin, hair wool or fur.

30. Use according to any one of Claims 27, 28 and 29 wherein the phosphorylation of serine residue 505 or 509 is inhibited.

31. Use according to Claim 29 or Claim 30 wherein the agent comprises a peptide that specifically interferes with the protein kinase C-beta-mediated phosphorylation of a serine or a threonine residue in the cytoplasmic domain of tyrosinase when introduced into epidermal melanocytes.

32. Use according to Claim 27 or Claim 31 wherein the peptide comprises an amino acid sequence homologous to the amino acid sequence comprising the site of the cytoplasmic domain of tyrosinase where protein kinase C-beta-mediated phosphorylation occurs.

33. Use according to Claim 32 wherein the peptide comprises from 5 to 30 amino acid residues and at least one of the residues is a serine.

34. Use according to Claim 33 wherein the peptide comprises an amino acid sequence selected from SEQ ID NO: 1 and SEQ ID NO: 4.

35. Use according to Claim 35 wherein the medicament is a pharmaceutical composition containing the peptide for introduction into the epidermal melanocytes by topical administration.

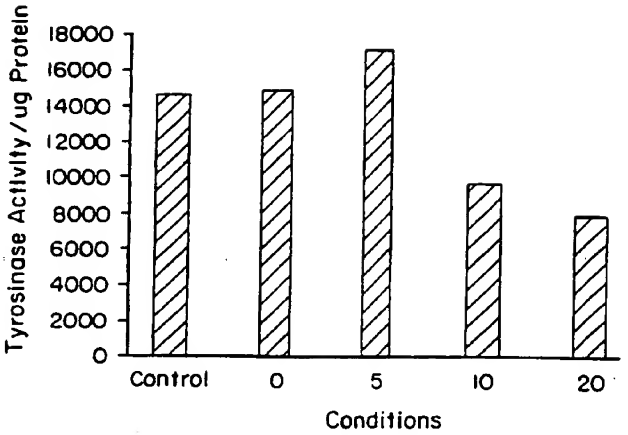
36. Use of a DNA construct for the manufacture of a medicament for decreasing pigmentation in vertebrate skin, hair, wool or fur by inhibiting the phosphorylation of tyrosinase in vertebrate epidermal melanocytes contained in skin or hair, wool or fur bulbs the medicament comprising the DNA construct encapsulated into a liposome for topical application to the skin or hair, wool or fur bulbs of the vertebrate; wherein the DNA construct encodes a peptide comprising an amino acid sequence homologous to the amino acid sequence comprising the site of the cytoplasmic domain of tyrosinase where protein kinase C-beta-mediated phosphorylation occurs, whereby when the DNA construct is introduced into the melanocytes the peptide is expressed in the melanocytes, resulting in inhibition of the phosphorylation of tyrosinase.

37. Use of a peptide of the manufacture of a medicament for decreasing pigmentation in vertebrate skin, hair, wool or fur by inhibiting the phosphorylation of tyrosinase in vertebrate epidermal melanocytes contained in skin or hair, wool or fur bulbs the

medicament comprising the peptide encapsulated into a liposome for topical application to the skin or skin surrounding hair, wool or fur bulbs of the vertebrate, wherein the peptide comprises an amino acid sequence homologous to the amino acid sequence comprising the site of the cytoplasmic domain of tyrosinase wherein protein kinase C-beta-mediated phosphorylation occurs, whereby when the peptide is introduced into the melanocytes phosphorylation of tyrosinase is inhibited.

38. A substance that modulates the protein kinase C-beta-mediated phosphorylation of tyrosinase, for use in therapy comprising for example the alteration of pigmentation.

39. Use for cosmetic purposes, for example the alteration of pigmentation, of a substance that modulates the protein kinase C-beta-mediated phosphorylation of tyrosinase.


Tyrosinase Activity / ug Protein
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Control
0
5
10
20
Conditions

INTERNATIONAL SEARCH REPORT

International plication No
PCT/US 97/04752

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/88 A61K7/135 A61K7/48 D06L3/00 G01N33/53
G01N33/577

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K D06L G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 16, 5 June 1993, MD US, pages 11742-11749, XP002036373 H-Y PARK ET AL.: "The beta isoform of protein kinase C stimulates human melanogenesis by activating tyrosinase in pigment cells" cited in the application see the whole document ---	1-3, 9-11, 17-30,39
X	EP 0 679 660 A (POLA CHEMICAL INDUSTRIES) 2 November 1995 see the whole document ---	18-20,23
	-/--	

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 July 1997	18.08.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Masturzo, P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US 97/04752

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 120, no. 25, 20 June 1994 Columbus, Ohio, US; abstract no. 320132z, XP002036376 see abstract & BRE. J. DERMATOL. , vol. 125, no. 4, 1991, pages 297-303, J M NAEYAERT ET AL.: "Pigment content of cultured human melanocytes does not correlate with tyrosinase message level" ---	1-3, 9-11, 17-30,39
X	BIOLOGICAL ABSTRACTS, vol. 83, 1982 Philadelphia, PA, US; abstract no. 168663, XP002036374 see abstract & PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 79, no. 6, 1982, WASHINGTON US, pages 2018-2022, M EISINGER & O MARKO: "Selective proliferation of normal human melanocytes in vitro in the presence of phorbol ester and cholera toxin" ---	38
X	BIOLOGICAL ABSTRACTS, vol. 90, 1989 Philadelphia, PA, US; abstract no. 4743, XP002036375 see abstract & J. INVEST. DERMATOL., vol. 93, no. 5, 1989, pages 700-702, P R GORDON & B A GILCHREST: "Human melanogenesis is stimulated by diacylglycerol" ---	38
X,P	CHEMICAL ABSTRACTS, vol. 125, no. 19, 4 November 1996 Columbus, Ohio, US; abstract no. 238942, XP002036377 see abstract & EXP. CELL RES., vol. 227, no. 1, 1996, pages 70-79, H Y PARK ET AL.: "Alpha-melanocyte stimulating hormone-induced pigmentation is blocked by depletion of protein kinase C" -----	1-39

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interna nal application No.
PCT/US 97/04752

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-17
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1 to 17 refer (at least in principle) to a method of treatment of the human body, the search has been carried out and based on the alleged effects of the compound.

2. [X] Claims Nos.: 35
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Claim 35 should be read as depending on claim 34, instead of being dependent on itself.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US 97/04752

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 679660 A	02-11-95	AU 7863794 A	06-06-95
		WO 9514042 A	26-05-95